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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/00

A2

(11) International Publication Number: WO 99/55901

(43) International Publication Date: 4 November 1999 (04.11.99)

(21) International Application Number: PCT/US99/08923

(22) International Filing Date: 23 April 1999 (23.04.99)

(30) Priority Data:
60/083,579 30 April 1998 (30.04.98) US

(71) Applicant: ABBOTT LABORATORIES [US/US]; CHAD-0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-6050 (US).

(72) Inventors: VANBIESEN, Tim; 4711 N. Dover Street, Chicago, IL 60640 (US). JARVIS, Michael, F.; 356 S. Old Wood Court, Vernon Hills, IL 60061 (US). LYNCH, Kevin, J.; 17904 West Hampshire Drive, Gurnee, IL 60031 (US). METZGER, Randy, E.; 225 Saratoga Court, Gurnee, IL 60031 (US). BURGARD, Edward, C.; 609 Burdick Street, Libertyville, IL 60048 (US).

(74) Agents: GOLLER, Mimi, C. et al.; Abbott Laboratories, CHAD 0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US).

(81) Designated States: CA, JP, MX, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: HIGH-CAPACITY FUNCTIONAL SCREENING ASSAY FOR IDENTIFYING HUMAN PURINORECEPTOR LIGANDS

(57) Abstract

A method of screening candidate compounds for their ability to modulate expression of human purinoreceptors is provided. The method relates to the use of stably transfected cell lines that express human or other mammalian purinoreceptors to identify purinoreceptor ligands, and to determine the amount of a receptor agonist or antagonist in a test sample.

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HIGH-CAPACITY FUNCTIONAL SCREENING ASSAY FOR IDENTIFYING HUMAN PURINORECEPTOR LIGANDS

Technical Field

The invention relates generally to receptor ligand screening methods. In particular, the invention relates to a method of using stably transfected cell lines that express human purinoreceptors to identify purinoreceptor ligands.

Background of the Invention

P2 purinoreceptors have been generally categorized as either metabotropic nucleotide receptors or ionotropic receptors for extracellular nucleotides. Metabotropic nucleotide receptors, designated $P2Y_n$, and the ionotropic receptors, designated $P2X_n$, are distinguished on the basis of their respective transmembrane signal transduction mechanisms as well as structural differences; the $P2Y_n$ receptors operate through a G protein-coupled system, while the $P2X_n$ receptors are ligand-gated ion channels. The ligand for these receptors may be ATP and/or another natural nucleotide such as ADP, UTP and UDP, or a synthetic nucleotides such as 2-methylthioATP.

At least seven P2X receptors, and the cDNA sequences therefore, have been identified to date. $P2X_1$ cDNA has been cloned from the smooth muscle of the rat vas deferens (Valera *et al.* (1994) *Nature* 371:516-519) and $P2X_2$ cDNA was cloned from PC12 cells (Brake *et al.* (1994) *Nature* 371:519-523). Five other P2X receptors have been found in rat neuronal cDNA libraries by virtue of their sequence similarity to $P2X_1$ and $P2X_2$ ($P2X_3$: Lewis *et al.* (1995) *Nature* 377:432-435, Chen *et al.* (1995) *Nature* 377:428-431; $P2X_4$: Buell *et al.* (1996) *EMBO J.* 15:55-62, Seguela *et al.* (1996) *J. Neurosci.* 16:448-455, Bo *et al.* (1995) *FEBS Lett.* 375:129-133, Soto *et al.* (1996) *Proc. Natl. Acad.*

Sci. USA 93:3684-3688, Wang *et al.* (1996) *Biochem. Biophys. Res. Commun.*220:196-202; $P2X_5$: Collo *et al.* (1996) *J. Neurosci.*16:2495-2507, Garcia-Guzman *et al.* (1996) *FEBS Lett.* 388:123-127; $P2X_4$: Collo *et al.* (1996), *supra*, Soto *et al.* (1996) *Biochem. Biophys. Res. Commun.* 223:456-460; $P2X_7$ Surprenant *et al.* (1996) *Science* 272:735-738. For a comparison of the amino acid sequences of rat P2X receptors see Buell *et al.* (1996) *Eur. J. Neurosci.* 8:2221-2228.

Native P2X receptors form rapidly-activated, nonselective cationic channels that are activated by ATP. $P2X_1$ and $P2X_2$ have equal permeability to Na^+ and K^+ but significantly less to Cs^+. The channels formed by the P2X receptors generally have high Ca^{2+} permeability (P_{Ca}/P_{Na} 4). The cloned rat $P2X_1$, $P2X_2$, and $P2X_4$ receptors exhibit the same permeability for Ca^{2+} observed with native receptors. However, the mechanism by which P2X receptors form an ionic pore or bind ATP is not known.

A variety of tissues and cell types, including epithelial, immune, muscle and neuronal, express at least one form of P2X receptor. (As there appear to be heteromeric as well as homomeric P2X receptors in certain tissues, and without intending to be bound by theory, it is believed that some cells in fact express two or more receptor forms.) Moreover, the association of particular receptor types with certain tissues suggests a functional specialization for some of these receptors. For example, the widespread distribution of $P2X_4$ receptors in the rat central nervous system suggests a role for $P2X_4$-mediated events in the central nervous system

Unfortunately, study of the role of individual P2X receptors is hampered by the lack of receptor subtype-specific agonists and antagonists. One agonist useful for studying ATP-gated channels is , -methylene-ATP (, meATP); however, the P2X receptors display differential sensitivity to the agonist with $P2X_1$ and $P2X_2$ being , meATP-sensitive and insensitive, respectively. Furthermore, binding of , meATP to P2X receptors does not always result in channel opening. The predominant forms of P2X receptors in the rat brain, $P2X_4$ and $P2X_6$ receptors, cannot be blocked by suramin or pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid ("PPADS"). These two forms of the P2X receptor are

also not activated by , meATP and are, thus, intractable to study with currently available pharmacological tools.

Similarly, a variety of P2Y receptors have been identified and cloned from tissues such as erythroleukemia cells ($P2Y_1$), airway epithelium ($P2Y_2$ and $P2Y_6$), and placenta ($P2Y_4$).

A potential therapeutic role for P2 purinoreceptors has been suggested, e.g., for cystic fibrosis (Boucher *et al.* (1995) in: Belardinelli *et al.* (eds.) *Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology* (Kluwer Acad., Norwell MA) pp 525-532), diabetes (Loubatiéres-Mariani *et al.* (1995) in: Belardinelli *et al.* (eds), *supra*, pp 337-345, immune and inflammatory diseases (Di Virgilio *et al.* (1995) in: Belardinelli *et al.* (eds), *supra*, pp 329-335), cancer (Rapaport (1993) *Drug Dev. Res.* 28:428-431), constipation and diarrhea (Milner *et al.* (1994) in: Kamm *et al.* (eds.) *Constipation and Related Disorders: Pathophysiology and Management in Adults and Children* (Wrightson Biomedical, Bristol) pp 41-49), behavioral disorders such as epilepsy, depression and aging-associated degenerative diseases (Williams (1993) *Drug. Dev. Res.* 28:438-444), contraception and sterility (Foresta *et al.* (1992) *J. Biol. Chem.* 257:19443-19447) and wound healing (Wang *et al.* (1990) *Biochim. Biophys. Res. Commun.* 166:251-258). There additionally may be possibilities in the treatment of pain, particularly in connection with $P2X_3$ homomeric receptors and $P2X_2/X_3$ heteromeric receptors.

Accordingly, for both research and therapeutic purposes there is a need in the art for specific agonists and antagonists for each purinoreceptor subtype and, in particular, agents that will be effective *in vivo*, as well as for methods for identifying purinoreceptor-specific agonist and antagonist compounds.

Summary of the Invention

The present invention relates to a method of identifying compounds that modulate the receptor or other therapeutic compounds using such cells. The method herein offers a variety of advantages, in that it (a) provides a means of distinguishing, during screening of compounds, between receptor agonists and antagonists; (b) exhibits greater

sensitivity than conventional methodologies, especially with respect to P2Y receptors and known phosphoinositide hydrolysis assays; and/or (c) is suitable for testing all P2 receptor agonists over a broad range of ligand concentrations.

In one embodiment, the invention is directed to a method for identifying compounds that modulate the activity of a purinoreceptor selected from the group consisting of human P2X and P2Y purinoreceptors. The method comprises (a) providing a cell which is a purinoreceptor null cell in its native non-transformed state and which comprises and expresses a polynucleotide encoding a human purinoreceptor polypeptide; (b)mixing a test compound with the cell; and (c) measuring either (i) the effect of the test compound on the activation of the human purinoreceptor or the cell expressing the purinoreceptor receptor, or (ii) the binding of the test compound to the cell or the receptor.

In another embodiment, the invention relates to a method for determining the amount of a receptor agonist or antagonist in a test sample. The method comprises (a) providing a cell that expresses a purinoreceptor polypeptide coding sequence, (b) mixing a the cell with a test sample, and (c) measuring the effect of the test compound on the activation of the purinoreceptor or the cell expressing the purinoreceptor receptor.

The purinoreceptors so expressed and utilized for testing may comprise either P2X or P2Y receptor subunits or, in the case of heteromeric constructs, both. Preferred types of P2X receptor include $P2X_2$, $P2X_3$ and P2X7, as well as $P2X_2$/X3 heteromers, with $P2X_3$ being particularly favored. Preferred types of P2Y receptor include $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$, with $P2Y_2$ being particularly favored.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein. In particular, while the description and examples which follow are drawn to the cloning, expression and testing of human purinoreceptors, it is anticipated that the method of the present invention may be carried out using analogous mammalian receptors from non-human sources, whose sequences and/or functional properties are similar enough to those of the corresponding human receptors as to be readily substituted therefor without undue experimentation.

Such non-human, and especially rat, P2 receptor clones may in such instances be regarded as equivalents to their human counterparts.

Brief Description of the Drawings

Figure 1 depicts the sequence of the intact open reading frame of the human P2X 4 receptor (SEQ ID NO:1). The EcoRI sites used in subcloning are underlined and the start (ATG) and stop (TGA) codons of the open reading frame ("ORF") are shaded.

Figure 2A is the Genbank submission (Accession No.U07225) of the human P2Y 2 receptor DNA sequence described in Example 4 (SEQ ID NO:2), in which the regions used for sense and antisense primer design are underlined. Figure 2B is the Genbank submission (same accession number) of the wild type human $P2Y_2$ amino acid sequence (SEQ ID NO:3).

Figure 3A is the cDNA sequence (SEQ ID NO:4) of the $P2Y_2$ clone prepared as described in Example 4, in which the primer sequences are underlined and the start (ATG) and stop (TGA) codons of the open reading frame are shown in bold italics. Figure 3B is the amino acid sequence (SEQ ID NO:5) of the $P2Y_2$ receptor encoded by the cDNA sequence depicted in Figure 3A.

Figure 4 is the cDNA sequence (SEQ ID NO:6) of the $P2X_3$ clone prepared as described in Example 7.

Figure 5 is the sequence of the RACE product of Example 7B (SEQ ID NO:23), including the EcoRl sites from the TA vector, in which the sequences of the amplimers (universal amplification primer and the complement to 5'RACE primer 4a) are underlined and the predicted termination codon is in bold.

Figure 6 is the sequence of the RACE product of Example 7C (SEQ ID NO:24), including the EcoRl sites from the TA vector, in which the sequences of the amplimers (universal amplification primer and the complement to 3'RACE primer 2s) are underlined and the predicted termination codon is in bold.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, electrophysiology, and pharmacology, that are within the skill of the art. Such techniques are explained fully in the literature. *See*, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Transcription and Translation* (Hames *et al.* eds. 1984); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller *et al.* eds. (1987) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); Scopes, *Protein Purification: Principles and Practice* (2nd ed., Springer-Verlag); and *PCR: A Practical Approach* (McPherson *et al.* eds. (1991) IRL Press).

All patents, patent applications and publications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, reference to "a primer" includes two or more such primers, reference to "an amino acid" includes more than one such amino acid, and the like.

A. DEFINITIONS

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms "P2 receptor" intends a purinergic receptor for the ligand ATP and/or other purine or pyrimidine nucleotides, natural or synthetic. P2 receptors are broadly subclassified as "P2X" or "P2Y" receptors. These types differ in their pharmacology, structure, and signal transduction mechanisms. The P2X receptors are generally ligand-gated ion channels, while the P2Y receptors operate generally through a G protein-coupled system. Moreover, and unless otherwise specified, the term "P2 receptor" is

further intended to mean both homomeric (consisting of one or more identical subunits) and heteromeric (comprising two or more different P2X subunits) receptors. Without intending to be limited by theory, such homomers and heteromers are believed to exist *in vivo* but may also be expressed by the cloning and transfection methods described below.

The term "subunit" when used in reference to purinoreceptors intends a polypeptide which, either alone or in combinantion with one or more other polypeptides, forms a functional purinoreceptor. Where a purinoreceptor comprises more than one polypeptide subunit, the subunits may be either identical (forming a homomeric multimer) or different (forming a heteromeric multimer).

The term "$P2X_n$" intends a P2X receptor subtype wherein n is an integer of one or more. At the time of the invention, at least 7 human $P2X_n$ receptor subtypes have been isolated and/or characterized.

The term "$P2Y_n$" intends a P2Y receptor subtype wherein n is an integer of one or more. At the time of the invention, at least 4 human $P2Y_n$ receptor subtypes have been isolated and/or characterized.

A "$P2X_n$ receptor agonist" or a "$P2Y_n$ receptor agonist" is a compound that binds to and activates a $P2X_n$ receptor or a $P2Y_n$ receptor, respectively. By "activates" is intended the elicitation of one or more pharmacological, physiological, or electrophysiological responses. Such a response includes, but is not limited to, an increase in receptor-specific cellular depolarization or increase in intracellular calcium levels due to calcium ion influx for a $P2X_n$ receptor, or an increase in intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) and/or inositolphospholipid hydrolysis and the formation of inositol phosphate for a $P2Y_n$ receptor.

A "$P2X_n$ receptor antagonist" or a "$P2Y_n$ receptor antagonist" is a substance that binds to a $P2X_n$ receptor or a $P2Y_n$ receptor, respectively, and prevents agonists from activating the receptor. Pure antagonists do not activate the receptor, but some substances may have mixed agonist and antagonist properties.

The term "polynucleotide" as used herein means a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, the term includes double- and single-stranded DNA, as well as double- and single-stranded RNA. It also includes modifications, such as by methylation and/or by capping, and unmodified forms of the polynucleotide.

The term "variant" is used to refer to an oligonucleotide sequence which differs from the related wild-type sequence in one or more nucleotides. Such a variant oligonucleotide is expressed as a "protein variant" which, as used herein, indicates a polypeptide sequence that differs from the wild-type polypeptide in the substitution, insertion or deletion of one or more amino acids. A protein variant differs in primary structure (amino acid sequence), but may or may not differ significantly in secondary or tertiary structure or in function relative to the wild-type.

The term "mutant" generally refers to an organism or a cell displaying a new genetic character or phenotype as the result of change in its gene or chromosome. In some instances, however, "mutant" may be used in reference to a variant protein or oligonucleotide and "mutation" may refer to the change underlying the variant.

"Polypeptide" and "protein" are used interchangeably herein and indicate a molecular chain of amino acids linked through peptide bonds. The terms do not refer to a specific length of the product. Thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. The terms include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. In addition, protein fragments, analogs, mutated or variant proteins, fusion proteins and the like are included within the meaning of polypeptide.

A "functionally conservative mutation" as used herein intends a change in a polynucleotide encoding a derivative polypeptide in which the activity is not substantially altered compared to that of the polypeptide from which the derivative is made. Such derivatives may have, for example, amino acid insertions, deletions, or substitutions in the relevant molecule that do not substantially affect its properties. For example, the derivative can include conservative amino acid substitutions, such as

substitutions which preserve the general charge, hydrophobicity/hydrophilicity, side chain moiety, and/or stearic bulk of the amino acid substituted, for example, Gly/Ala, Val/Ile/Leu, Asp/Glu, Lys/Arg, Asn/Gln, Thr/Ser, and Phe/Trp/Tyr.

By the term "structurally conservative mutant" is intended a polynucleotide containing changes in the nucleic acid sequence but encoding a polypeptide having the same amino acid sequence as the polypeptide encoded by the polynucleotide from which the degenerate variant is derived. This can occur because a specific amino acid may be encoded by more than one "codon," or sequence of three nucleotides, i.e., because of the degeneracy of the genetic code.

"Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, immaterial of the method by which the DNA is introduced into the cell or the subsequent disposition of the cell. The terms include the progeny of the original cell which has been transfected. Cells in primary culture as well as cells such as oocytes also can be used as recipients.

A "null" host cell is a host cell that does not express a purinoreceptor of interest within the limits of the methods used to measure the expression of such receptors, either pharmacologically, electrophysiologically, biochemically, or the like. Thus, a P2X-P2Y null cell, that is, a "$P2X^{-}$-$P2Y^{-}$ host cell," is a cell in which the presence of neither the P2X receptor nor the P2Y receptor can be measured. This characteristic of the null host cell may be due to mutation of a gene that otherwise naturally encodes a P2X or P2Y receptor such that the mutant encodes a purinoreceptor polypeptide that is not detectable by methods used to detect the native P2X or P2Y receptor. On the other hand, a null host cell may not express a purinoreceptor polypeptide to any measurable extent. The definition of "null" host cell is not intended to be limited to any particular mechanism underlying the absence of measurable levels of a purinoreceptor.

A "vector" is a replicon in which another polynucleotide segment is attached, such as to bring about the replication and/or expression of the attached segment. The term includes expression vectors, cloning vectors, and the like.

A "coding sequence" is a polynucleotide sequence that is transcribed into mRNA and/or translated into a polypeptide. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, mRNA, cDNA, and recombinant polynucleotide sequences. Variants or analogs may be prepared by the deletion of a portion of the coding sequence, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. *See, e.g.*, Sambrook *et al.*, *supra*; *DNA Cloning*, Vols. I and II, *supra*; *Nucleic Acid Hybridization*, *supra*.

"Operably linked" refers to a situation wherein the components described are in a relationship permitting them to function in their intended manner. Thus, for example, a control sequence "operably linked" to a coding sequence is ligated in such a manner that expression of the coding sequence is achieved under conditions compatible with the control sequences. A coding sequence may be operably linked to control sequences that direct the transcription of the polynucleotide whereby said polynucleotide is expressed in a host cell.

The term "transfection" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, or the molecular form of the polynucleotide that is inserted. The insertion of a polynucleotide *per se* and the insertion of a plasmid or vector comprised of the exogenous polynucleotide are included. The exogenous polynucleotide may be directly transcribed and translated by the cell, maintained as a nonintegrated vector, for example, a plasmid, or alternatively, may be stably integrated into the host genome. "Transfection" generally is used in reference to a eukaryotic cell while the term "transformation" is used to refer to the insertion of a polynucleotide into a prokaryotic cell. "Transformation" of a eukaryotic cell also may refer to the formation of a cancerous or tumorigenic state.

The term "isolated," when referring to a polynucleotide or a polypeptide, intends that the indicated molecule is present in the substantial absence of other similar biological macromolecules. The term "isolated" as used herein means that at least 75

wt.%, more preferably at least 85 wt.%, more preferably still at least 95 wt.%, and most preferably at least 98 wt.% of a composition is the isolated polynucleotide or polypeptide. An "isolated polynucleotide" that encodes a particular polypeptide refers to a polynucleotide that is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include functionally and/or structurally conservative mutations as defined herein.

A "test sample" as used herein intends a component of an individual's body which is a source of a purinoreceptor. These test samples include biological samples which can be evaluated by the methods of the present invention described herein and include body fluids such as whole blood, serum, tissues and cell preparations.

The following single-letter amino acid abbreviations are used throughout the text:

Alanine	A	Arginine	R
Asparagine	N	Aspartic acid	D
Cysteine	C	Glutamine	Q
Glutamic acid	E	Glycine	G
Histidine	H	Isoleucine	I
Leucine	L	Lysine	K
Methionine	M	Phenylalanine	F
Proline	P	Serine	S
Threonine	T	Tryptophan	W
Tyrosine	Y	Valine	V

B. General Methods

The present invention provides a method for screening a plurality of compounds for specific binding to a purinoreceptor to identify a compound that modulates the activity of the receptor. The method comprises (a) providing a cell that expresses the human (or other mammalian) purinoreceptor polypeptide coding sequence, (b) mixing a test compound with the cell, and (c) measuring the effect of the test compound on the activation of the purinoreceptor or the cell expressing the purinoreceptor receptor.

In addition, the invention provides a method for determining the amount of a receptor agonist or antagonist in a test sample. The method comprises (a) providing a cell that expresses the human (or other mammalian) purinoreceptor polypeptide coding sequence, (b) mixing a the cell with a test sample, and (c) measuring the effect of the test compound on the activation of the purinoreceptor or the cell expressing the purinoreceptor receptor.

The invention disclosed and claimed herein comprises providing a host cell that encodes the purinoreceptor of interest. The host cell is genetically engineered with a vector, which may be a cloning vector or an expression vector, comprising a polynucleotide sequence encoding a purinoreceptor operably linked to control sequences that control its expression. Preferably, the host cell is stably transfected to express the purinoreceptor. More preferably, the host cell is a purinoreceptor null cell which, if not already lacking endogenous purinoreceptor expression, has been so engineered.

The vector may be in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants/transfectants or amplifying the subunit-encoding polynucleotide. The culture conditions, such as temperature, pH and the like, generally are similar to those previously used with the host cell selected for expression, and will be apparent to those of skill in the art.

Either a prokaryotic or a eukaryotic host cell may be used for expression of desired coding sequences when appropriate control sequences that are compatible with the designated host are used. Preferably the host cell is a null host cell, more preferably, when the expression of the coding sequence result in production of a P2X purinoreceptor polypeptide, the host cell is any $P2X^-$ cell or a cell in which expression of P2X purinoreceptors is less than can be detectably measured. When a P2Y purinoreceptor is produced from expression of the coding sequence, the host cell is preferably a $P2Y^-$ null cell or a cell in which expression of P2Y purinoreceptors is less than can be detectably measured; optionally, but not necessarily, the host cell used to express a P2Y purinoreceptor may be 1321N1 (human astrocytoma) or another $P2X^-$-$P2Y^-$ null cell.

Among prokaryotic hosts, *Escherichia coli* is frequently used. Expression control sequences for prokaryotic host cells include but are not limited to promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts can be derived from, for example, the plasmid pBR322 that contains operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, that also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Commonly used prokaryotic control sequences include but are not limited to the lactose operon system (Chang *et al.* (1977) *Nature* 198:1056), the tryptophan operon system (reported by Goeddel *et al.* (1980) *Nucleic Acid Res.* 8:4057) and the lambda-derived Pl promoter and N gene ribosome binding site (Shimatake *et al.* (1981) *Nature* 292:128), the hybrid *Tac* promoter (De Boer *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 292:128) derived from sequences of the *trp* and *lac* UV5 promoters. The foregoing systems are particularly compatible with *E. coli*; however, other prokaryotic hosts such as strains of *Bacillus* or *Pseudomonas* may be used if desired.

Eukaryotic hosts include yeast and mammalian cells in culture systems. *Pichia pastoris, Saccharomyces cerevisiae* and *S. carlsbergensis* are commonly used yeast hosts. Yeast-compatible vectors carry markers that permit selection of successful transformants by conferring protrophy to auxotrophic mutants or resistance to heavy metals on wild-type strains. Yeast-compatible vectors may employ the 2-μ origin of replication (Broach *et al.* (1983) *Meth. Enzymol.* 101:307), the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences that will result in incorporation of an appropriate fragment into the host cell genome. Control sequences for yeast vectors are known in the art and include but are not limited to promoters for the synthesis of glycolytic enzymes, including the promoter for 3-phosphoglycerate kinase. *See*, for example, Hess *et al.* (1968) *J. Adv. Enzyme Reg.* 7:149, Holland *et al.* (1978) *Biochemistry* 17:4900 and Hitzeman (1980) *J. Biol. Chem.* 255:2073. For example, some useful control systems are those that comprise the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter or alcohol dehydrogenase (ADH) regulatable promoter, or the hybrid yeast promoter

ADH2/GAPDH described in Cousens *et al. Gene* (1987) 61:265-275, terminators also derived from GAPDH, and, if secretion is desired, leader sequences from yeast alpha factor. In addition, the transcriptional regulatory region and the transcriptional initiation region which are operably linked may be such that they are not naturally associated in the wild-type organism.

Mammalian cell lines available as hosts for expression are known in the art and are available from depositories such as the American Type Culture Collection. These include but are not limited to HeLa cells, human embryonic kidney (HEK) cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, and others. Suitable promoters for mammalian cells also are known in the art and include viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV) and cytomegalovirus (CMV). Mammalian cells also may require terminator sequences and poly A addition sequences; enhancer sequences which increase expression also may be included, and sequences which cause amplification of the gene also may be desirable. These sequences are known in the art. Vectors suitable for replication in mammalian cells may include viral replicons, or sequences which ensure integration of the appropriate sequences encoding the purinoreceptor into the host genome. An example of such a mammalian expression system is described in Gopalakrishnan *et al.* (1995), *Eur. J. Pharmacol.* 290: 237-246.]

Other eukaryotic systems are also known, as are methods for introducing polynucleotides into such systems, such as amphibian cells, using standard methods such as described in Briggs *et al.* (1995) *Neuropharmacol.* 34:583-590 or Stühmer (1992) *Meth. Enzymol.* 207:319-345, insect cells using methods described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), and the like.

The baculovirus expression system can be used to generate high levels of recombinant proteins in insect host cells. This system allows for high level of protein expression, while post-translationally processing the protein in a manner similar to mammalian cells. These expression systems use viral promoters that are activated following baculovirus infection to drive expression of cloned genes in the insect cells

(O'Reilly *et al.* (1992) Baculovirus Expression Vectors: A Laboratory Manual, IRL/Oxford University Press).

DNA encoding the purinoreceptor of interest can be derived from genomic or cDNA, prepared by synthesis, or by a combination of techniques. The DNA can then be used to express the purinoreceptor or as a template for the preparation of RNA using methods well known in the art (*see*, Sambrook *et al.*, *supra*). For example, the human $P2X_1$ purinoreceptor cDNA may be obtained as described in International Publication Number WO 95/33048, while the human $P2Y_1$ receptor cDNA may be obtained as described in Léon *et al.* (1966) *Gene* 171:295-297.

cDNA encoding a $P2X_n$ receptor or a $P2Y_n$ receptor may be obtained from an appropriate DNA library. cDNA libraries may be probed using the procedure described by Grunstein *et al.* (1975) *Proc. Natl. Acad. Sci. USA* 73:3961. The cDNA thus obtained can then be modified and amplified using the polymerase chain reaction ("PCR") and primer sequences to obtain the DNA encoding the desired $P2X_n$ or $P2Y_n$ receptor. Alternatively, the wild-type DNA may be obtained from an appropriate DNA library. DNA libraries may be probed using the procedure described by Grunstein *et al.* (1975) *Proc. Natl. Acad. Sci. USA* 73:3961. The wild-type cDNA thus obtained can then modified and amplified using PCR and mutated primer sequences to obtain the DNA encoding the receptor of interest.

More particularly, PCR employs short oligonucleotide primers (generally 10-20 nucleotides in length) that match opposite ends of a desired sequence within the DNA molecule. The sequence between the primers need not be known. The initial template can be either RNA or DNA. If RNA is used, it is first reverse transcribed to cDNA. The cDNA is then denatured, using well known techniques such as heat, and appropriate oligonucleotide primers are added in molar excess.

Alternatively, DNA encoding a human (or other mammalian) $P2X_n$ or $P2Y_n$ receptor can be derived from genomic or cDNA, prepared by synthesis, or by a combination of techniques. The DNA can then be used to express the receptor or as a template for the preparation of RNA using methods well known in the art (*see*,

Sambrook *et al.*, *supra*). An example of a method for obtaining the desired DNA involves isolating cDNA encoding the wild-type human $P2Y_2$ receptor as described by Parr *et al.* (1994), *supra*.

Primer extension is effected using DNA polymerase in the presence of deoxynucleotide triphosphates or nucleotide analogs. The resulting product includes the respective primers at their 5'-termini, covalently linked to the newly synthesized complements of the original strands. The replicated molecule is again denatured, hybridized with primers, and so on, until the product is sufficiently amplified. Such PCR methods are described in, for example, U.S. Patent Nos. 4,965,188, 4,800,159, 4,683,202 and 4,683,195, and are believed to be well-known to the skilled artisan. The product of the PCR is cloned and the clones containing the $P2X_4$ receptor DNA, derived by segregation of the primer extended strand, selected. Selection can be accomplished using a primer as a hybridization probe.

Alternatively still, the $P2X_n$ receptor or $P2Y_n$ receptor DNA could be generated using an RT-PCR (reverse transcriptase - polymerase chain reaction) approach starting with RNA. The RNA may be obtained from cells or tissue in which the $P2X_n$ or $P2Y_n$ receptor is expressed, e.g., brain, spinal cord, uterus or lung, using conventional methods. For example, single-stranded cDNA is synthesized from RNA as the template using standard reverse transcriptase procedures and the cDNA is amplified using. This is but one example of the generation of a $P2X_n$ or $P2Y_n$ receptor from a mammalian tissue RNA template.

Synthetic oligonucleotides may be prepared using an automated oligonucleotide synthesizer such as that described by Warner (1984) *DNA* 3:401. If desired, the synthetic strands may be labeled with ^{32}P by treatment with polynucleotide kinase in the presence of ^{32}P-ATP, using standard conditions for the reaction. DNA sequences, including those isolated from genomic or cDNA libraries, may be modified by known methods which include site-directed mutagenesis as described by Zoller (1982) *Nucleic Acids Res.* 10:6487. Briefly, the DNA to be modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase

using, as a primer, a synthetic oligonucleotide complementary to the portion of the DNA to be modified, and having the desired modification included in its own sequence. Culture of the transformed bacteria, which contain replications of each strand of the phage, are plated in agar to obtain plaques. Theoretically, 50% of the new plaques contain phage having the mutated sequence, and the remaining 50% have the original sequence. Replicates of the plaques are hybridized to labeled synthetic probe at temperatures and conditions suitable for hybridization with the correct strand, but not with the unmodified sequence. The sequences which have been identified by hybridization are recovered and cloned. Alternatively, it may be necessary to identify clones by sequence analysis if there is difficulty in distinguishing the variant from wild-type by hybridization. In any case, the DNA would be sequence-confirmed.

Once produced, DNA encoding the $P2X_n$ or $P2Y_n$ purinoreceptor may then be incorporated into a cloning vector or an expression vector for replication in a suitable host cell. Vector construction employs methods known in the art. Generally, site-specific DNA cleavage is performed by treating with suitable restriction enzymes under conditions that generally are specified by the manufacturer of these commercially available enzymes. After incubation with the restriction enzyme, protein is removed by extraction and the DNA recovered by precipitation. The cleaved fragments may be separated using, for example, polyacrylamide or agarose gel electrophoresis methods, according to methods known by those of skill in the art.

Sticky end cleavage fragments may be blunt ended using *E. coli* DNA polymerase 1 (Klenow) in the presence of the appropriate deoxynucleotide triphosphates (dNTPs) present in the mixture. Treatment with S1 nuclease also may be used, resulting in the hydrolysis of any single stranded DNA portions.

Ligations are performed using standard buffer and temperature conditions using T4 DNA ligase and ATP. Alternatively, restriction enzyme digestion of unwanted fragments can be used to prevent ligation.

Standard vector constructions generally include specific antibiotic resistance elements. Ligation mixtures are transformed into a suitable host, and successful transformants selected by antibiotic resistance or other markers. Plasmids from the

transformants can then be prepared according to methods known to those in the art usually following a chloramphenicol amplification as reported by Clewell *et al.* (1972) *J. Bacteriol.* 110:667. The DNA is isolated and analyzed usually by restriction enzyme analysis and/or sequencing. Sequencing may be by the well-known dideoxy method of Sanger *et al.* (1977) *Proc. Natl. Acad. Sci. USA* 74:5463) as further described by Messing *et al.* (1981) *Nucleic Acid Res.* 9:309, or by the method reported by Maxam *et al.* (1980) *Meth. Enzymol.* 65:499. Problems with band compression, which are sometimes observed in GC rich regions, are overcome by use of, for example, T-deazoguanosine or inosine, according to the method reported by Barr *et al.* (1986) *Biotechniques* 4:428.

Transfection may be by any known method for introducing polynucleotides into a host cell, including packaging the polynucleotide in a virus and transducing a host cell with the virus, by direct uptake of the polynucleotide by the host cell, and the like, which methods are known to those skilled in the art. The transfection procedures selected depend upon the host to be transfected and are determined by the rountineer.

The expression of the purinoreceptor may be detected by use of a radioligand selective for the receptor. For example, such ligands include [^{35}S]ATP S, [^{35}S]ATP S, [^{35}S]ATP S, [^{3}H]ATP and [^{3}H] meATP (*see*, Michel *et al.* (1997) *Mol. Pharmacol.* 51:524-532). However, any radioligand binding technique known in the art may be used to detect the receptor (*see, e.g.*, Winzor *et al.* (1995) *Quantitative Characterization of Ligand Binding*, Wiley-Liss, Inc., NY). Alternatively, expression can be detected by utilizing antibodies or functional measurements, i.e., ATP- or UTP-stimulated cellular depolarization using methods that are well known to those skilled in the art. For example, agonist-stimulated Ca^{2+} influx, or inhibition by antagonists of agonist-stimulated Ca^{2+} influx, can be measured in mammalian cells that express endogenous and/or recombinant P2 receptor, such as HEK, CHO, COS and PC12 (rat pheochromocytoma) cells. In a particular embodiment of such methods, Ca^{2+} influx can be measured in cells that do not naturally express any P2 receptor (such as the 1321N1

human astrocytoma cell line) but have been prepared using recombinant technology to transiently or stably express a human P2X or P2Y purinoreceptor.

In one method of expression, DNA which encodes a P2X or P2Y purinoreceptor, or messenger RNA derived therefrom, may be introduced by direct injection into a cell such as a *Xenopus laevis* oocyte. Using this method, the functionality of the purinoreceptor encoded by the DNA or the mRNA can be evaluated as follows. A receptor-encoding polynucleotide is injected into an oocyte for translation into a functional receptor subunit. The function of the expressed human purinoreceptor can be assessed in the oocyte by a variety of techniques including electrophysiological techniques such as voltage-clamping (*see, e.g.*, Briggs *et al.* (1995), *supra*) and the like.

Receptors expressed in a recombinant host cell may be used to identify compounds that bind to and/or modulate the activity of a purinoreceptor. In this regard, the specificity of the binding of a compound showing affinity for the receptor is demonstrated by measuring the affinity of the compound for cells expressing the receptor or membranes from these cells. This may be done by measuring specific binding of labeled (*e.g.*, radioactive) compound to the cells, cell membranes or isolated receptor, or by measuring the ability of the compound to displace the specific binding of a standard labeled ligand. *See,* Michel *et al.*, *supra*. Expression of variant receptors and screening for compounds that bind to, or inhibit the binding of labeled ligand to these cells or membranes, provide a method for rapid selection of compounds with high affinity for the receptor. These compounds may be agonists, antagonists or modulators of the receptor.

Expressed receptors also may be used to screen for compounds that modulate purinoreceptor activity. One method for identifying compounds that modulate human purinoreceptor activity, comprises providing a cell that expresses a human (or, if analogous, other mammalian) purinoreceptor polypeptide, combining a test compound with the cell and measuring the effect of the test compound on the purinoreceptor activity. The cell may be a bacterial cell, a mammalian cell, a yeast cell, an amphibian cell, an insect cell or any other cell expressing the receptor. Preferably, the cell is a mammalian cell or an amphibian cell, more preferably the cell is a purinoreceptor null

cell as described above. Thus, for example, a test compound is evaluated for its ability to elicit an appropriate response, e.g., the stimulation of cellular depolarization or increase in intracellular calcium levels due to calcium ion influx if a P2X purinoreceptor is expressed in the host cell, the stimulation of an increase in intracellular calcium ion levels and/or inositolphospholipid hydrolysis and the formation of inositol phosphate if a P2Y purinoreceptor is expressed, or for the compound's ability to modulate the response to a P2X or P2Y purinoreceptor agonist or antagonist.

The level of intracellular calcium may be analyzed using a calcium ion-sensitive fluorescent indicator. Cellular fluorescence may be monitored using a fluorometer. Examples of calcium ion-sensitive fluorescent dyes include, for example, quin-2 (*see, e.g.*, Tsien *et al.* (1982) *J. Cell. Biol.* 94:325), fura-2 (*see, e.g.*, Grynkiewicz *et al.* (1985) *J. Biol. Chem.* 260:3440), calcium green-1, indo-1 (*see, e.g.*, Grynkiewicz *et al.*, *supra*), fluo-3 (*see, e.g.*, Kao *et al.* (1989) *J. Biol. Chem.* 264:8179) and rhod-2 (*see, e.g.*, Tsien *et al.*(1987) *J. Biol. Chem.* abstract 89a), and the nonspecific esterase-hydrolyzable acetoxymethyl esters thereof, all of which are commercially available (Molecular Probes, Eugene, OR; Sigma Chemical Co., St. Louis, MO).

Membrane depolarization of cells genetically engineered to express a $P2X_n$ purinoreceptor may be monitored using a fluorescent dye that is sensitive to changes in membrane potential. For example, the potential-sensitive fluorescent dye partitions into a membrane upon depolarization and results in a detectable increase in cellular fluorescence. Examples of such membrane potential-sensitive fluorescent dyes include carbocyanines, such as 3,3'-dipentyloxacarbocyanine iodide ($DiOC_5$) and 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3$), oxonols, such as bis-(1,3-dibutylbarbituric acid)pentamethine oxonol ($DiBAC_4(5)$) or bis-(1,3-dibutylbarbituric acid)pentamethine oxonol ($DiBAC_4(5)$), or the like.

In order to calibrate the fluorescence emission of these dyes *in situ*, an agent that quenches the fluorescence emission may be used. Thus, for example, anti-fluorescein (Molecular Probes) quenches approximately 87% of the fluorescence of a 5 nM solution of fluo-3 at pH 7.0, and may used to calibrate the fluorescence emission of this dye.

When acetoxymethyl ester dye derivatives are use, incomplete hydrolysis of the ester may result in a fluorescent indicator that is flourescent but insensitive to calcium ions. Controls for such a situation include transporting saturating amounts of calcium ions into the cell by an ionophore to achieve the maximum fluorescence response and transport of manganese ions into the cell to quench the fluorescence of the indicator if all acetoxymethyl esters have been hydrolyzed. One means by which such ions can be transported into cells is with the use of an ionophore, such as A23187 (*see, e.g.*, Pressman *et al.* (1976) *Ann. Rev. Biochem.* 45:501) (Sigma Chemical Co.), the brominated derivative thereof (*see, e.g.*, Deber *et al.* (1985) *Anal. Biochem.* 146:349) (Molecuar Probes), or other ionophores well known in the art.

In addition, it may be desirable to quantify the amount of intracellular calcium ion from the fluorescence emission of a cell by comparing the fluorescence data obtained from the test compounds to a calibration curve that was generating by a series of calibrators each having a known calcium ion concentration. Thus, calcium ion standards are made having a range of concentrations bu preparing a stock solution of, e.g., $CaCl_2$, from which dilutions may be made to attain the desired standard concentration(s).

the indicator dye and the calcium ion standards used to generate the standard curve.

The assay may be conducted manually or using an automated system. For a high capacity functional screening assay identifying human purinoreceptor ligands, an automated system is preferred. An example of such an automated system comprises providing a 96-well culture plate in each well of which is cultured a cell genetically engineered to encode and express a human purinoreceptor polypeptide. The plate is loaded into a fluorescence imaging plate reader ("FLIPR"), which simultaneously measures the kinetics of intracellular calcium flux in each of the 96 wells. Such an FLIPR is commercially available from Molecular Devices Corp. (Sunnyvale, CA). The FLIPR is capable of quantitatively transferring fluids into and from each well of the 96-well plate and thus can be used to add the calcium-ion sensitive fluorescent indicator dye, a candidate compound, a purinoreceptor agonist, e.g., ATP, UTP, 2-methylthioATP, or the like, and/or a purinoreceptor antagonist, e.g., suramin, cibacron

blue, PPADS, or the like. The FLIPR collects fluorescence data throughout the course of the assay.

In a similar manner, the presence of a purinoreceptor agonist or antagonist in a test sample may be determined using a manual or an automated system. An automated system for practicing the method comprises providing a 96-well culture plate in each well of which a genetically engineered cell that expresses a purinoreceptor is cultured. The fluorescent indicator dye, test sample, and/or purinoreceptor agonist are added to each well and the fluorescence emission from each well is simultaneously monitored by an FLIPR.

P2X purinoreceptor drugs are considered potential therapeutic agents in several disorders including, without limitation, central nervous system or peripheral nervous system conditions, e.g., epilepsy, pain, depression, neurodegenerative diseases, and the like, and in disorders of the reproductive system, asthma, peripheral vascular disease, hypertension, immune system disorders, irritable bowel disorder or premature ejaculation.

P2Y purinoreceptors are believed to mediate the activity of extracellular nucleotide triphosphates to regulate chloride secretion in human airway epithelia. Cystic fibrosis exhibits reduced chloride secretion by airway epithelia and, consequently, dehydrated, viscous mucus that obstructs airways and compromises pulmonary function. Thus, drugs that are able to regulate epithelia chloride secretion may provide an alternative non-CFTR-dependent mechanism to induce fluid secretion in cystic fibrosis airways. In addition, extracellular nucleotides stimulate mucus secretion by goblet cells *in vitro* and excessive activation of this pathway *in vivo* may be partly responsible for the hypersecretion observed in chronic bronchitis. Thus, drugs that regulate this pathway may have therapeutic value in chronic bronchitis.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1

Cloning of Human P2X$_4$ cDNA into pcDNA3.1

A. Identification of a Human Sequence With Homology to P2X Receptors

The predicted amino acid sequence of the rat P2X$_4$ receptor (Genbank accession #X91200) was used to search for human DNA sequences that would code for similar polypeptides. The TBLASTN database search tool (Altschul (1993) *J. Mol. Evol.* 36:290-300) was used, which allows querying nucleotide databases with a protein sequence by dynamically translating the DNA sequences into all six possible reading frames. A search of the Lifeseq database (Incyte Pharmaceuticals, Inc., Palo Alto, CA) revealed a partial sequence of a cDNA clone derived from human knee synovium from a patient with rheumatoid arthritis encoding a polypeptide with a high degree of homology to a region of the rat P2X$_4$ receptor. The database entry for this sequence (Incyte clone #1260936) is shown below:

GCGGGNCCATGGCGGGCTGCTGCGCCGCGCTGGNGCCCTTTCCTGTTCGAGT
ACGACACGCCGCGCATCGTGCTCATCCGCAGCCGCAAAGTGGGGCTCATGAA
CCGCGCCGTGCAACTGCTCATCCTGGCCTACGTCATCGGGT (SEQ ID NO:7)
(N = A, T, G, or C)

The position of this sequence with respect to the rat P2X$_4$ sequence predicted that this cDNA clone should contain the entire coding sequence for the human P2X$_4$ receptor. The cDNA clone was ordered from Incyte. Additional clones from the Incyte database (#555697, 095809, 705958, and 711949) and the Genbank dbEST database (#60722) also aligned to the rat P2X$_4$ receptor sequence, and information from these sequences

was compiled and used to design primers for PCR amplification of the intact open reading frame (ORF) for the human P2X$_4$ receptor from clone 1260936. The sequence immediately upstream of the initiation codon of the ORF was modified in the design of the primers to incorporate a consensus translation initiation signal to optimize gene expression (Kozak (1984) *Nucl. Acids Res.* 12:857-872). The sequences of the two human P2X$_4$ primers are: 5'-GCG<u>CCA</u>CCATGGCGGGCTGCTGCGCCGCGCTG-3' (sense) (SEQ ID NO:8) and 5'-GGTAGGCCTCACTGGTCCAGCTCACTAGCAAG-3' (antisense) (SEQ ID NO:9).

B. Subcloning of the ORF for the Human P2X$_4$ Receptors

To facilitate transfer of the open reading frame of the human P2X$_4$ receptor, polymerase chain amplification reactions were used on the Incyte clone 1260936 using the primers designed from the consensus sequence of the predicted 5' and 3' ends of the open reading frame. Template DNA (100 ng) was used in a 100 μl amplification reaction that also included 200 μM dNTPs, 15 pmoles each primer, 10 μl 10X GeneAmp PCR buffer (Perkin Elmer, Foster City, CA) (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, and 0.01% (w/v) gelatin), and 0.5 μl (2.5 units) Amplitaq® polymerase (Perkin Elmer). The reaction was cycled 35 times in a Perkin Elmer Model 9600 thermocycler under the following conditions: 95 C for 30 sec, 55 C for 30 sec, and 72 C for 2 min. The predominant product of 1.2 kilobase pairs was isolated by electrophoresis on a 1% low melting point agarose gel and purified. A portion of the purified product (10%) was used in a ligation reaction with the vector pCRII (Invitrogen, Carlsbad, CA) using the manufacturer's protocols. The ligation products were used to transform competent DH5-alpha *E. coli*. Resulting clones were screened by restriction analysis and a representative clone was sequenced using fluorescent dye-terminator reagents (Prism, Perkin Elmer-Applied Biosystems Division) and a Perkin Elmer-Applied Biosystems Model 373 DNA sequencer. The insert from this clone was excised from the vector using the restriction enzyme *Eco*RI and was used in a ligation reaction with the mammalian expression vector pCDNA3.1(+) (Strategene, La Jolla, CA).

Reaction products were used to transform competent *E. coli* and resulting clones were screened by restriction analysis. A representative clone was sequenced. The sequence of the insert is shown in Figure 1. To guard against sequence errors introduced by the DNA amplification process, the ORF contained in the original Incyte clone 1260936 was also sequenced and found to be identical to that of the expression clone.

Example 2

Stable Transfection of 1321N1 Cells with $P2X_4$ cDNA

Human astrocytoma cells, 1321N1, were grown to 80% confluence in 35-mm wells in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were incubated at 37 C in a humidified atmosphere containing 5% CO_2. Human $P2X_4$ cDNA cloned into pcDNA3.1 as described in Example 1 was transfected into the 1321N1 cells using standard lipid-mediated transfection methodology. Briefly, 2 μg of plasmid DNA was preincubated with 12 μl LipofectAMINE (Life Technologies) in a 1 ml volume of serum-free DMEM for 30 minutes ate room temperature. The 1321N1 cells were washed with serum-free DMEM before the addition of the transfection medium. Cells were incubated with the transfection medium for 3 hours at 37 C. The transfection medium was aspirated and fresh DMEM + 10% FBS was added). After 48 hours incubation at 37 C, the cells in a 35-mm dish were split into 5 150-mm plates. The plates were incubated for an additional 7-10 days at 37 C in DMEM + 10% FBS + 800 μg/ml Geneticin (G418 antibiotic, Life Technologies). This step selects for cells in which the transfected plasmid has been stably integrated into the host chromosome.

Individual clones were grown to confluence in a 24-well plate in DMEM + 10% FBS + 300 μg/ml Geneticin. These clones were subsequently screened for functional $P2X_4$ expression using the calcium influx assay on an FLIPR instrument. Several positive clones were identified; one clone, 1321X4-15, was selected for further characterization and for use in the high-throughput screening assay.

Example 3

Screening Assay For P2X4 Receptor Ligands

1321X4-15 cells are grown to confluence in black-walled 96-well tissue culture plates in DMEM + 10% FBS + 300 μg/ml Geneticin. A Fluo-3 AM (Molecular Probes) solution is prepared by dispersing 40 μl of a 1 mg/ml stock DMSO solution into 10 ml Dulbecco's phosphate-buffered saline (D-PBS). The growth medium is aspirated from the cells and the Fluo-3 AM suspension is added. Cells are incubated in the dark for 2 hours at room temperature. In all cases, the tissue culture plates are handled with care because physical agitation might cause the release of endogenous ATP from the cells, thereby activating the $P2X_4$ receptors prior to the assay. Cells are washed gently three times with D-PBS on a Denley Cellwash instrument. The Cellwash leaves a final volume of 100 μl D-PBS in each well.

Three different plates are loaded into a fluorescence imaging plate reader ("FLIPR") for the assay: (1) the 1321X4-15 cell plate (washed); (2) a source plate of test compounds (at 4 times the desired final concentration); and (3) a plate containing approximately 4 times the final concentration of any $P2X_4$ receptor agonist. The cells are assayed in the FLIPR as follows. All pipetting steps are performed by the FLIPR's built-in pipetting armature: 50 μl test compound is added to the cell plate 10 seconds after the start of the run; and 50 μl agonist is added 3 min after the start of the run. The FLIPR instrument collects fluorescence data throughout the course of the run.

Example 4

Cloning of Human $P2Y_2$ cDNA into pcDNA3.1

The DNA sequence and amino acid sequence of the reported human $P2Y_2$ (P2U) receptor depicted in Figure 2A (SEQ ID NO:2) and Figure 2B (SEQ ID NO:3), respectively, were retrieved from the Genbank database. Amplification primers were designed to amplify the intact open reading frame (ORF) contained in the $P2Y_2$ nucleotide sequence. The sequence immediately upstream of the initiation codon was modified in the design of the primers to incorporate a consensus translation initial signal to optimize recombinant gene expression (Kozak (1984) *Nucl. Acids Res.* 12:857-872). Flanking sense and antisense primers, respectively, used to subclone the human $P2Y_2$ open reading frame, in which the initiation (ATG) and termination (TAG-opposite strand) are underlined, 5'-GCGCGGTACCCACC<u>ATG</u>GCAGCAGACCTGGGC-3' (SEQ ID NO:10) and 5'-CTACGACGTCTAGA<u>CTA</u>CAGCCGAATGTCCTT-3' (SEQ ID NO:11).

A reverse-transcription reaction was performed using standard methods. The 20 μl reaction mixture contained: 2 μg of total RNA from human placental tissue; 4 μl 25 mN $MgCl_2$, 2 μl 10X PCR Buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3, and 0.01% (w/v) gelatin) (Perkin Elmer, Foster City, CA); 2 μl each 10 mM dATP, dGTP, dCTP, and dTTP; 1 μl RNAse inhibitor (Perkin Elmer); 1 μl 10 mM oligo $d(T)_{16}$ primer; and 1 μl MuLV reverse transcriptase. The reaction was incubated for 10 min at room temperature, 42 C for 15 min, 99 C for 5 min, and 5 C for 5 min. The resulting cDNA was precipitated by adding 2.5 μl of 10 M NH_4OAc and 60 μl 100% ethanol, incubated at -20 C for 1 hr, and centrifuged for 15 min at 4 C. The DNA thus obtained was washed with 70% ethanol and dried *in vacuo*. The reverse transcriptase products were suspended in 81.5 μl distilled water.

The PCR amplification reaction consisted of the reverse transcriptase products, 10 μl 10X PCR Buffer II, 4 μl 25 mM $MgCl_2$, 2 μl each 10 mM dATP, dGTP, dCTP, and dTTP, 15 picomoles each $P2Y_2$ primer, and 1.5 μl Amplitaq® polymerase (Perkin

Elmer). The reaction was cycled 30 times in a Perkin-Elmer model 9600 thermocycler under the following parameters: 95 C, 30 sec; 50 C, 30 sec; and 72 C, 2 min. The major reaction product of 1.1 kilobase pairs was purified via low melting point agarose electrophoresis, and ligated into the vector pCR2.1 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The products were used to transform DH5-alpha competent *E.coli* and resulting clones were screened via restriction analysis. The insert from a representative clone was excised by digestion with *Eco*RI and inserted into the corresponding site of the mammalian expression vector pCDNA3.1 (+). The ligation products were used to transform DH5-alpha competent *E. coli* and resulting clones were screened via restriction analysis. A representative clone was chosen for sequencing via fluorescent dye-terminator reagents (Prism, Perkin Elmer-Applied Biosystems Division) and a Perkin Elmer-Applied Biosystems Model 373 DNA sequencer. The sequence of this clone is shown in Figure 3A (SEQ ID NO:4), and the predicted amino acid sequence encoded thereby is depicted in Figure 3B (SEQ ID NO:5). Six differences were observed between the sequence in Figure 3 and the Genbank submission. These differences would result in three amino acid differences between the predicted polypeptides: R (Genbank) C at position 334; G E at position 350; and F S at position 359. The encoded receptors have been shown to exhibit identical functional characteristics.

EXAMPLE 5

Stable Transfection of 1321N1 Cells With $P2Y_2$ cDNA

The stably transfected 1321Y2-8 cell line was constructed as described in Example 2 substituting the $P2Y_2$ cDNA prepared as described in Example 4 for the P2X $_4$ cDNA.

Example 6

Screening Assay For $P2Y_2$ Receptor Ligands

The assay for $P2Y_2$ receptor ligands is conducted as described in Example 3 for $P2X_4$ receptor ligands with the following difference: a 1321Y2-8 cell plate is substituted for the 1321X4-15 cell plate, and the third plate loaded into the FLIPR contains an appropriate amount of the P2 agonist.

Example 7

Cloning of Human $P2X_3$ cDNA into pcDNA3.1

A. Identification of a human genomic sequence with homology to P2X receptors.

The predicted amino acid sequence of the rat $P2X_3$ receptor (NCBI seq. I.D. 1103623) was used to search for human DNA sequences which would code for similar polypeptides. The TBLASTN database search tool (Altschul (1993) *J. Mol. Evol.* 36:290-300) was used, which allows querying nucleotide databases with a protein sequence by dynamically translating the DNA sequences into all 6 possible reading frames. A search of the Genbank sequence-tagged sites (STS) database revealed a human genomic fragment containing an open reading frame which encodes a polypeptide with a high degree of homology to a region of the rat $P2X_3$ receptor (Genbank accession number G03901). Primers were designed based on the sequence of G03901 for use in reverse-transcription polymerase chain reaction (RT-PCR) procedures in an effort to isolate the intact open reading frame for this receptor. The primers used in the reactions described below are as follows:

5'TTTACCAACCCAGTGTACCC3' ($hP2X_3$-1S) (SEQ ID NO:12);

5'ACCACAGTGGAGAAGCAGTC3' ($hP2X_3$-2S) (SEQ ID NO:13);

5'GAATCGGTGGACTGCTTCTC3' ($hP2X_3$-3AS) (SEQ ID NO:14);

5'CGATTTTCAGTGTAGTCTCATTC3' ($hP2X_3$-4AS) (SEQ ID NO:15);

5'GGGGTACACTGGGTTGGTAA3' ($hP2X_3$-5AS) (SEQ ID NO:16);

5'CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG3' (5' RACE Anchor Primer) (SEQ ID NO:17);

5'CUACUACUACUAGGCCACGCGTCGACTAGTAC3' (Universal Adapter Primer) (SEQ ID NO:18);

5'GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTT3' (Adapter Primer) (SEQ ID NO:19);

5'GGCCACGCGTCGACTAGTAC3' (Abridged Universal Adapter Primer) (SEQ ID NO:20);

5'CACCATGAACTGCATATCCGACTTC3' (5'$hP2X_3$ Primer) (SEQ ID NO:21); and

5'CTAGTGGCCTATGGAGAAGGC3' (3'$hP2X_3$ Primer) (SEQ ID NO:22).

B. Identification of the 5' end of the $P2X_3$ cDNA.

To identify the 5' end of the cDNA which is derived from the genomic region which sequence G03901 is part of, the RACE technique (Rapid Amplification of cDNA Ends) (Frohman *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85:8998-9002) was employed. Extension of the cDNA identified through the RT-PCR step was accomplished using the 5'RACE reagent system (Life Technologies). One microgram of poly A+ RNA derived from human pituitary gland tissue (Cat. # 65894-1, Lot #

6080167; Clontech Laboratories, Palo Alto, CA) was used in a reaction using reagents provided in the kit as described; 1 μl (1 μg) of RNA was combined with 3 μl (3 pmol) primer 3as and 11 μl DEPC-treated water and heated to 70 C for 10 min. followed by 1 min. on ice. 2.5 μl 10X reaction buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 3 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTP mix, and 2.5 μl 0.1 M DTT were added. The mix was incubated at 42 C for 2 min. after which 1 μl Superscript II reverse transcriptase was added. The reaction was incubated for an additional 30 min. at 42 C, 15 min. at 70 C, and on ice for 1 min. One microliter of RNase H (2 units) was added and incubated at 55 C for 20 min. The cDNA was purified using the GlassMax columns included in the kit. The cDNA was eluted from the column in 50 μl dH_20, lyophilized, and resuspended in 21 μl dH_2O. Tailing of the cDNA was accomplished in the following reaction: 7.5 μl dH_2O, 2.5 μl reaction buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 μl 25 mm $MgCl_2$, 2.5 μl 2 mM dCTP, and 10 μl of the cDNA were incubated at 94 C for 3 min., then 1 min. on ice, followed by 10 min. at 37 C. Finally, the mixture was incubated at 70 C for 10 min. and then placed on ice. PCR amplification of the cDNA was performed in the following steps: 5 μl of the cDNA was included in a reaction which also contained 5 μl 10x GeneAmp PCR buffer (Perkin-Elmer) (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, and 0.01%(w/v) gelatin), 1 μl 10 mM dNTP mix, 1 μl (10 pmol) anchor primer, 1 μl (10 pmol) primer 2A, and 35 μl dH_20. The reaction was heated to 95 C for 1 min., then held at 80 C for 2 min., during which 0.5 μl (2.5 units) Amplitaq polymerase was added. The reaction was cycled 35 times under these conditions; 94 C for 15 sec., 52 C for 20 sec., and 72 C for 1 min. After the amplification, the reaction products were purified utilizing the Cacique PCR product purification system (Qiagen,Inc., Chatsworth CA) as per manufacturer's instructions. The products were eluted from the columns with 50 μl TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) and one microliter of the eluent was utilized as template DNA in a PCR reaction to increase levels of specific product for subsequent isolation. The reamplification also included: 5 μl 10x GeneAmp PCR buffer, 1 μl 10 mM dNTP mix, 1 μl (10 pmol) universal amplification primer, 1 μl (10 pmol) primer 4as, and 40.5 μl dH_2

O. The reaction was heated to 95 C for 1 min., then held at 80 C during which 0.5 μl (2.5 units) Amplitaq polymerase was added. The reaction was cycled 35 times under these conditions; 94 C for 15 sec., 50 C for 20 sec., and 72 C for 1 min. Amplification products were analyzed via 0.8% agarose gel electrophoresis and a predominant product of approximately 1.3 kilobase pairs in length was detected. This product was excised from the gel and purified via the Cacique purification system. The product was eluted from the column with 50 μl dH_2O and lyophilized to 10 μl volume. Three microliters of this DNA was used in a ligation reaction with pCR 2.1 vector (Invitrogen, Carlsbad, CA)) incubated at 14 C overnight. The ligation products were used to transform *E. coli* from the cloning kit using standard manufacturer's protocols. Insert sizes of resulting clones were determined using EcoRI digestions of the plasmids and clones containing inserts of the approximate size of the PCR product were sequenced using fluorescent dye-terminator reagents (Prism, Applied Biosystems) and an Applied Biosystems 373 DNA sequencer. The sequence of the RACE product (SEQ ID NO:23), including the EcoRI sites from the TA vector, is shown in Figure 5.

C. Identification of the 3'end of the $P2X_3$ cDNA

To identify the sequence surrounding the termination codon of the open reading frame encoding the human $P2X_3$, receptor, the Life Technologies 3'RACE System was employed with primers designed to STS G03901. Poly A+ RNA (500 nanograms) derived from pituitary gland tissue was used in the reaction as follows: The RNA and 10 picomoles primer AP were combined in a final volume of 12 μl dH_2O. This mixture was heated to 70 C for 10 min. and chilled on ice for 1 min. The following components were added: 2 μl 10X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTP mix, and 2 μl 0.1 M dithiothreitol. The reaction was equilibrated to 42 C for 2 min. after which 1 μl (200 units) Superscript II reverse transcriptase was added and incubation continued at 42 C for 50 minutes. The reaction was terminated by incubation at 70 C for 15 min. and chilled on ice. Rnase H (1 μl; 2

units) was added and the mixture was incubated for 20 minutes at 37 C, then stored on ice.

Amplification of the 3' end of the $P2X_3$ cDNA was accomplished in the following reactions: 2 μl of the first strand cDNA synthesized above was used in a PCR mixture also including 5 μl 10X GeneAmp PCR buffer, 1 μl 10 mM dNTPs, 1 μl (10 picomoles) $P2X_3$ primer 1s, 1 μl (10 picomoles) abridged universal amplification primer (AUAP) and 39.5 μl dH_2O. The reaction was heated to 95 C for 1 min., then held at 80 C for 2 min., during which 0.5 μl (2.5 units) Amplitaq polymerase was added. The reaction was cycled 35 times under these conditions; 94 C for 15 sec., 54 C for 20 sec., and 72 C for 2 min. After cycling, the reaction was incubated for 10 minutes at 70 C and stored at 4 C. After the amplification, the reaction products were purified utilizing the QiaQuick PCR product purification system as per manufacturer's instructions. The products were eluted from the columns with 50 μl TE buffer (10 mM Tris, 0.1 mM EDTA pH 8.0) and one microliter of the eluent was utilized as template DNA in a PCR reaction to increase levels of specific product for subsequent isolation. The reamplification also included: 5 μl 10X GeneAmp PCR buffer, 1 μl 10 mm dNTP mix, 1 μl (10 pmol) AUAP, 1 μl (10 pmol) primer 2s, and 40.5 μl dH_2O. The reaction was heated to 95 C for 1 min., then held at 80 C during which 0.5 μl (2.5 units) Amplitaq polymerase was added. The reaction was cycled 35 times under these conditions; 94 C for 15 sec., 54 C for 20 sec., and 72 C for 2 min. Amplification products were analyzed via 0.8% agarose gel electrophoresis and a predominant product of approximately 700 base pairs in length was detected. This product was excised from the gel and purified via the Qiaquick purification system. The product was eluted from the column with 50 μl dH_2O and lyophilized to 10 μl volume. Three microliters of this DNA was used in a ligation reaction with pCR 2.1 vector (Invitrogen, Carlsbad, CA)) incubated at 15 C for 3.5 hours. The ligation products were used to transform *E. coli* from the cloning kit. Insert sizes of resulting clones were determined using EcoRI digestions of the plasmids and clones containing inserts of the approximate size of the PCR product were sequenced using fluorescent dye-terminator reagents (Prism, Applied

Biosystems) and an Applied Biosystems 373 DNA sequencer. The sequence of the RACE product (SEQ ID NO:24), including the EcoRI sites from the TA vector, are shown in Figure 6.

D. Isolation of cDNA containing the intact open reading frame for human $P2X_3$

Using information on the sequence surrounding the initiation and termination codons of the human $P2X_3$, message, oligonucleotide primers were designed and synthesized to enable RT-PCR of the intact open reading frame of the mRNA. The sequence of these primers, $hP2X_35'$ and $hP2X_33'$, are given above. PCR amplification was performed on a portion (2 μl) of the pituitary gland cDNA described above. A proofreading thermostable polymerase (Cloned *Pfu* DNA Polymerase, Stratagene) was used in the amplification to ensure high-fidelity amplification. The reaction mixture consisted of. 2 μl cDNA, 5 μl 10X cloned *Pfu* polymerase reaction buffer (200 mM Tris-HCl (pH 8.8), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/ml nuclease-free bovine serum albumin), 1 μl dNTP mix, 1 μl (10 picomoles) $5'hP2X_3$ primer, 1 μl (10 picomoles) $3'hP2X_3$ primer, and 39.5 μl dH_20. The reaction was heated to 95 C for 1 min., then held at 80 C for 2 min., during which 0.5 μl (1.25 units) cloned *Pfu* polymerase was added. The reaction was cycled 35 times under these conditions; 94 C for 20 sec., 52 C for 20 sec., and 72 C for 3.5 min. After cycling, the reaction was incubated for 10 minutes at 70 C. The reaction products were separated on a 0.8 % agarose gel and a product of approximately 1.2 kilobases was excised and purified via the Qiaquick gel purification system. The DNA was eluted with 50 μl dH_2 O, lyophilized and resuspended in 10 μl dH_2O. One microliter of this DNA was use in a reamplification reaction which also included 5 μl 10X Pfu reaction buffer, 1 μl dNTP mix, 1 μl (10 picomoles) $5'hP2X_3$ primer, 1 μl (10 picomoles) $3'hP2X_3$ primer, and 40.5 μl dH_2O. The reaction was heated to 95 C for 1 min., then held at 80 C for 2 min., during which 0.5 μl (1.25 units) cloned *Pfu* polymerase was added. The reaction was cycled 15 times under these conditions; 94 C for 20 sec., 52 C for 20 sec., and 72 C for 3.5 min. After cycling, the reaction was incubated for 10 minutes at 70 C. The

reaction products were separated on a 0.8 % agarose gel and the 1.2 kilobase product was excised and purified via the Qiaquick gel purification system. The DNA was eluted with 50 μl dH_20, lyophilized and resuspended in 15 μl dH_2O. Three microliters of the purified PCR product was used in a ligation reaction using the pCRscript cloning system (Stratagene)which also included: 0.5 μl (5 ng) of the pCRScript Amp SK(+) vector, 1 μl of pCRScript 10X Reaction Buffer, 0.5 μl of 10 mM ATP, 1 μl (5 units) *Srf* I restriction enzyme, 1 μl (4 units) T4 DNA ligase, and 3 μl dH_2O. The reaction was incubated at room temperature for one hour, then at 65 C for 10 minutes. One microliter of this reaction mix was used to transform XL-2 blue ultracompetent cells (Stratagene) as per standard manufacturer's protocols. Resulting clones were screened by restriction analysis and sequenced using fluorescent dye-terminator reagents (Prism, Applied Biosystems) and an Applied Biosystems 310 DNA sequencer. The sequence of the intact open reading frame (SEQ ID NO:6) is shown in Figure 4, in which the initiation codon (ATG) and termination codon (TAG) are in bold. 5' and 3' flanking sequences, including the EcoRI (GAATTC) and Not I (GCGGCCGC) restriction sites, is sequence introduced during plasmid construction and are underlined.

Example 8

Stable Transfection of 1321N1 Cells With $P2X_3$ cDNA

The stably transected 1321X3-11 cell line was constructed as described in Example 2, substituting $P2X_3$ cDNA prepared as described in Example 7 for the $P2X_4$ cDNA.

Example 9

Screening Assay For $P2X_3$ Purinoreceptor Ligands

The screening assay for $P2X_3$ receptor ligands is conducted as described in Example 3 for $P2X_4$ purinoreceptor ligands with the following differences: A 1321X3-

11 cell plate is substituted for the 1321X4-15 cell plate, and the third plate contains an appropriate amount of $P2X_3$ agonist.

Example 10

Cloning, Expression and Utilization of Other Purinoreceptors

Transformed host cells, and screening assays utilizing such cells, are prepared according to the procedures of the foregoing examples for the following human receptor clones:

Clone	Accession No.	Tissue Source
$hP2X_1$	X83688	urinary bladder
$hP2X_3$	Y07683	heart
$hP2X_4$	Y07684	brain
$hP2X_{M(6)}$	AB002058	skeletal muscle
$hP2X_7$	Y09561	brain
$hP2Y_1$	U42030	erythroleukemia cells
$hP2Y_2$	U07225	airway epithelium
$hP2Y_4$	X91852	placenta
$hP2Y_6$	X97058	airway epithelium

In each case, PCR primers are prepared according to the sequence information available at the respective GenBank accession number. The primers are then used to clone the DNA sequence of interest into a suitable cloning vector, following the procedures described in Examples 1 and 7 above. After stable transfection of the receptor DNA into appropriate host cells according to the procedures of Examples 2 and 8, screening assays for receptor ligands are conducted as described in Example 3 with any necessary modifications and/or substitutions.

What is claimed is:

1. A method for identifying compounds that modulate the activity of a mammalian purinoreceptor selected from the group consisting of P2X and P2Y receptors, comprising the steps of:

(a) providing a cell genetically engineered to comprise a polynucleotide encoding a polypeptide subunit of the mammalian purinoreceptor, the subunit being expressed by the cell so as to form a functional receptor;

(b) exposing the cell to a test compound; and

(c) detecting at least one of:

(i) an effect of the test compound on the activation of the receptor or on the cell expressing the receptor, and

(ii) binding of the test compound to the cell or the receptor.

2. The method of Claim 1, wherein the genetically engineered cell has been stably transfected with a vector comprising the polynucleotide encoding the purinoreceptor subunit.

3. The method of Claim 1, wherein the cell, in its native nontransformed state, is selected from the group consisting of $P2X^-$, $P2Y^-$ and $P2X^-$-$P2Y^-$ cells.

4. The method of any of Claims 1, 2 and 3, wherein the purinoreceptor subunit is a human purinoreceptor subunit.

5. The method of Claim 1, wherein the purinoreceptor subunit is selected from the group consisting of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ purinoreceptor subunits.

6. The method of Claim 1, wherein the purinoreceptor subunit is selected from the group consisting of $P2X_2$, $P2X_3$ and $P2X_7$ purinoreceptor subunits.

7. The method of Claim 1, wherein the purinoreceptor subunit is a $P2X_3$ purinoreceptor subunit.

8. The method of any of Claims 5, 6 and 7, wherein the purinoreceptor subunit is a human purinoreceptor subunit.

9. The method of any of Claims 5, 6 and 7, wherein the cell, in its native non-transformed state, is selected from the group consisting of a $P2X^-$ and $P2X^-$-$P2Y^-$ cells.

10. The method of Claim 1,wherein the purinoreceptor subunit is selected from the group consisting of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ purinoreceptor subunits.

11. The method of Claim 1, wherein the purinoreceptor subunit is a $P2Y_2$ purinoreceptor subunit.

12. The method of any of Claims 10 and 11, wherein the purinoreceptor subunit is a human purinoreceptor subunit.

13. The method of any of Claims 10 and 11, wherein the cell, in its native non-transformed state, is selected from the group consisting of a $P2Y^-$ and $P2X^-$-$P2Y^-$ cells.

14. The method of any of Claims 10 and 11, wherein the cell, in its native non-transformed state, is a $P2Y^-$ cell.

15. The method of claim 5, wherein the nucleotide encoding the $P2X_4$ purinoreceptor subunit comprises substantially all of the nucleotide sequence shown in Figure 1 (SEQ ID NO:1).

16. The method of claim 7, wherein the polynucleotide encoding the $P2X_3$ purinoreceptor subunit comprises substantially all of the nucleotide sequence shown in Figure 4 (SEQ ID NO:6).

17. The method of claim 11, wherein the nucleotide encoding the $P2Y_2$ purinoreceptor subunit comprises substantially all of the nucleotide sequence shown in Figure 3A (SEQ ID NO:4).

18. A method for determining the amount or activity of a purinoreceptor agonist or antagonist in a test sample, comprising:

(a) providing a cell genetically engineered to comprise a polynucleotide encoding a polypeptide subunit of a mammalian purinoreceptor, the subunit being expressed by the cell so as to form a functional receptor;

(b) exposing the cell to the test sample; and

(c) measuring at least one of:

(i) an effect of the test sample on the activation of the receptor or on the cell expressing the receptor, and

(ii) displacement of a ligand from the cell or the receptor by the test sample.

19. The method of Claim 18, wherein the genetically engineered cell has been stably transfected with a vector comprising the polynucleotide encoding the purinoreceptor subunit.

20. The method of Claim 18, wherein the cell, in its native nontransformed state, is selected from the group consisting of $P2X^-$, $P2Y^-$ and $P2X^-$-$P2Y^-$ cells.

21. The method of any of Claims 18, 19 and 20, wherein the purinoreceptor subunit is a human purinoreceptor subunit.

22. The method of Claim 18, wherein the purinoreceptor subunit is selected from the group consisting of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ purinoreceptor subunits.

23. The method of Claim 18, wherein the purinoreceptor subunit is selected from the group consisting of $P2X_2$, $P2X_3$ and $P2X_7$ purinoreceptor subunits.

24. The method of Claim 18, wherein the purinoreceptor subunit is a $P2X_3$ purinoreceptor subunit.

25. The method of any of Claims 22, 23 and 24, wherein the purinoreceptor subunit is a human purinoreceptor subunit.

26. The method of any of Claims 22, 23 and 24, wherein the cell, in its native non-transformed state, is selected from the group consisting of a $P2X^-$ and $P2X^-$-$P2Y^-$ cells.

27. The method of Claim 18,wherein the purinoreceptor subunit is selected from the group consisting of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ purinoreceptor subunits.

28. The method of Claim 18, wherein the purinoreceptor subunit is a $P2Y_2$ purinoreceptor subunit.

29. The method of any of Claims 27 and 28, wherein the purinoreceptor subunit is a human purinoreceptor subunit.

30. The method of any of Claims 27 and 28, wherein the cell, in its native non-transformed state, is selected from the group consisting of a $P2Y^-$ and $P2X^-$-$P2Y^-$ cells.

31. The method of any of Claims 27 and 28, wherein the cell, in its native non-transformed state, is a $P2Y^-$ cell.

32. The method of claim 22, wherein the nucleotide encoding the $P2X_4$ purinoreceptor subunit comprises substantially all of the nucleotide sequence shown in Figure 1 (SEQ ID NO:1).

33. The method of claim 24, wherein the polynucleotide encoding the $P2X_3$ purinoreceptor subunit comprises substantially all of the nucleotide sequence shown in Figure 4 (SEQ ID NO:6).

34. The method of claim 28, wherein the nucleotide encoding the $P2Y_2$ purinoreceptor subunit comprises substantially all of the nucleotide sequence shown in Figure 3A (SEQ ID NO:4).

GAATTCGGCTTTGCGCCACC**ATG**GCGGGCTGCTGCGCCGCGCTGGCGGCCTTC
CTGTTCGAGTACGACACGCCGCGCATCGTGCTCATCCGCAGCCGCAAAGTGGG
GCTCATGAACCGCGCCGTGCAACTGCTCATCCTGGCCTACGTCATCGGGTGGG
TGTTTGTGTGGGAAAAGGGCTACCAGGAAACTGACTCCGTGGTCAGCTCCGTT
ACGACCAAGGTCAAGGGCGTGGCTGTGACCAACACTTCTAAACTTGGATTFCCG
GATCTGGGATGTGGCGGATTATGTGATACCAGCTCAGGAGGAAAACTCCCTCT
TCGTCATGACCAACGTGATCCTCACCATGAACCAGACACAGGGCCTGTGCCCC
GAGATTCCAGATGCGACCACTGTGTGTAAATCAGATGCCAGCTGTACTGCCGG
CTCTGCCGGCACCCACAGCAACGGAGTCTCAACAGGCAGGTGCGTAGCTTTCA
ACGGGTCCGTCAAGACGTGTGAGGTGGCGGCCTGGTGCCCGGTGGAGGATGA
CACACACGTGCCACAACCTGCTTTTTAAAGGCTGCAGAAAACTTCACTCTTTTG
GTTAAGAACAACATCTGGTATCCCAAATTTAATTTCAGCAAGAGGAATATCCTTC
CCAACATCACCACTACTTACCTCAAGTCGTGCATTTATGATGCTAAAACAGATCC
CTTCTGCCCCATATTCCGTCTTGGCAAAATAGTGGAGAACGCAGGACACGGTTT
CCAGGACATGGCCGTGGAGGGAGGCATCATGGGCATCCAGGTCAACTGGGAC
TGCAACCTGGACAGAGCCGCCTCCCTCTGCTTGCCCAGGTACTCCTTCCGCCG
CCTCGATACACGGGACGTTGAGCACAACGTATCTCCTGGCTACAATTTCAGGTT
TGCCAAGTACTACAGAGACCTGGCTGGCAACGAGCAGCGCACGCTCATCAAGG
CCTATGGCATCCGCTTCGACATCATTGTGTTTGGGAAGGCAGGGAAATTTGACA
TCATCCCCACTATGATCAACATCGGCTCTGGCCTGGCACTGCTAGGCATGGCG
ACCGTGCTGTGTGACATCATAGTCCTCTACTGCATGAAGAAAAGACTCTACTAT
CGGGAGAAGAAATATAAATATGTGGAAGATTACGAGCAGGGTCTTGCTAGTGA
GCTGGACCAG**TGA**GGCCTACCAAGCCGAATTC (SEQ ID NO:1)

Figure 1

1 CGGCACGAGG CACCCCGAGA GGAGAAGCGC AGCGCAGTGG CGAGAGGAGC CCCTTGTGGC
61 AGCAGCACTA CCTGCCCAGA AAAATGCTGG AGGCTGGGCG TGGCCCCAGG CCTGGGGACC
121 TGTTTTTCCT GTTTCCCGCA GAGTTCCCTG CAGCCCGGTC CAGGTCCAGG CGTGTGCATT
181 CATGAGTGAG GAACCCGTGC AGGCGCTGAG CATCCTGACC TGGAGAGCAG GGGCTGGTCA
241 GGGCGATGGC AGCAGACCTG GGCCCCTGGA ATGACACCAT CAATGGCACC TGGGATGGGG
301 ATGAGCTGGG CTACAGGTGC CGCTTCAACG AGGACTTCAA GTACGTGCTG CTGCCTGTGT
361 CCTACGGCGT GGTGTGCGTG CTTGGGCTGT GTCTGAACGC CGTGGCGCTC TACATCTTCT
421 TGTGCCGCCT CAAGACCTGG AATGCGTCCA CCACATATAT GTTCCACCTG GCTGTGTCTG
481 ATGCACTGTA TGCGGCCTCC CTGCCGCTGC TGGTCTATTA CTACGCCCGC GGCGACCACT
541 GGCCCTTCAG CACGGTGCTC TGCAAGCTGG TGCGCTTCCT CTTCTACACC AACCTTTACT
601 GCAGCATCCT CTTCCTCACC TGCATCAGCG TGCACCGGTG TCTGGGCGTC TTACGACCTC
661 TGCGCTCCCT GCGCTGGGGC CGGGCCCGCT ACGCTCGCCG GGTGGCCGGG GCCGTGTGGG
721 TGTTGGTGCT GGCCTGCCAG GCCCCCGTGC TCTACTTTGT CACCACCAGC GCGCGCGGGG
781 GCCGCGTAAC CTGCCACGAC ACCTCGGCAC CCGAGCTCTT CAGCCGCTTC GTGGCCTACA
841 GCTCAGTCAT GCTGGGCCTG CTCTTCGCGG TGCCCTTTGC CGTCATCCTT GTCTGTTACG
901 TGCTCATGGC TCGGCGACTG CTAAAGCCAG CCTACGGGAC CTCGGGCGGC CTCCCTAGGG
961 CCAAGCGCAA GTCCGTGCGC ACCATCGCCG TGGTGCTGGC TGTCTTCGCC CTCTGCTTCC
1021 TGCCATTCCA CGTCACCCGC ACCCTCTACT ACTCCTTCCG CTCGCTGGAC CTCAGCTGCC
1081 ACACCCTCAA CGCCATCAAC ATGGCCTACA AGGTTACCCG GCCGCTGGCC AGTGCTAACA
1141 GTTGCCTTGA CCCCGTGCTC TACTTCCTGG CTGGGCAGAG GCTCGTACGC TTTGCCCGAG
1201 ATGCCAAGCC ACCCACTGGC CCCAGCCCTG CCACCCCGGC TCGCCGCAGG CTGGGCCTGC
1261 GCAGATCCGA CAGAACTGAC ATGCAGAGGA TAGGAGATGT GTTGGGCAGC AGTGAGGACT
1321 TCAGGCGGAC AGAGTCCACG CCGGCTGGTA GCGAGAACAC TAAGGACATT CGGCTGTAGG
1381 AGCAGAACAC TTCAGCCTGT GCAGGTTTAT ATTGGGAAGC TGTAGAGGAC CAGGACTTGT
1441 GCAGACGCCA CAGTCTCCCC AGATATGGAC CATCAGTGAC TCATGCTGGA TGACCCCATG
1501 CTCCGTCATT TGACAGGGGC TCAGGATATT CACTCTGTGG TCCAGAGTCA ACTGTTCCCA
1561 TAACCCCTAG TCATCGTTTG TGTGTATAAG TTGGGGGAAT TAAGTTTCAA GAAAGGCAAG
1621 AGCTCAAGGT CAATGACACC CCTGGCCTGA CTCCCATGCA AGTAGCTGGC TGTACTGCCA
1681 AGGTACCTAG GTTGGAGTCC AGCCTAATCA AGTCAAATGG AGAAACAGGC CCAGAGAGGA
1741 AGGTGGCTTA CCAAGATCAC ATACCAGAGT CTGGAGCTGA GCTACCTGGG GTGGGGGCCA
1801 AGTCACAGGT TGGCCAGAAA ACCCTGGTAA GTAATGAGGG CTGAGTTTGC ACAGTGGTCT
1861 GGAATGGACT GGGTGCCACG GTGGACTTAG CTCTGAGGAG TACCCCCAGC CCAAGAGATG
1921 AACATCTGGG GACTAATATC ATAGACCCAT CTGGAGGCTC CCATGGGCTA GGAGCAGTGT
1981 GAGGCTGTAA CTTATACTAA AGGTTGTGTT GCCTGCTAAA AAAAA (SEQ ID NO:2)

Figure 2A

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        10         20         30         40         50
MAADLGPWND TINGTWDGDE LGYRCRFNED FKYVLLPVSY GVVCVLGLCL

        60         70         80         90        100
NAVALYIFLC RLKTWNASTT YMFHLAVSDA LYAASLPLLV YYYARGDHWP

       110        120        130        140        150
FSTVLCKLVR FLFYTNLYCS ILFLTCISVH RCLGVLRPLR SLRWGRARYA

       160        170        180        190        200
RRVAGAVWVL VLACQAPVLY FVTTSARGGR VTCHDTSAPE LFSRFVAYSS

       210        220        230        240        250
VMLGLLFAVP FAVILVCYVL MARRLLKPAY GTSGGLPRAK RKSVRTIAVV

       260        270        280        290        300
LAVFALCFLP FHVTRTLYYS FRSLDLSCHT LNAINMAYKV TRPLASANSC

       310        320        330        340        350
LDPVLYFLAG QRLVRFARDA KPPTGPSPAT PARRRLGLRR SDRTDMQRIG

       360        370
DVLGSSEDFR RTESTPAGSE NTKDIRL   377   (SEQ ID NO:3)
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Figure 2B

GCGCGGTACCCACCATGGCAGCAGACCTGGGCCCCTGGAATGACACCATCAATGGCACCTGG
GATGGGGATGAGCTGGGCTACAGGTGCCGCTTCAACGAGGACTTCAAGTACGTGCTGCTGCCT
GTGTCCTACGGCGTGGTGTGCGTGCTTGGGCTGTGTCTGAACGCCGTGGCGCTCTACATCTTCT
TGTGCCGCCTCAAGACCTGGAATGCGTCCACCACATATATGTTCCACCTGGCTGTGTCTGATGC
ACTGTATGCGGCCTCCCTGCCGCTGCTGGTCTATTACTACGCCCGCGGCGACCACTGGCCCTT
CAGCACGGTGCTCTGCAAGCTGGTGCGCTTCCTCTTCTACACCAACCTTTACTGCAGCATCCTC
TTCCTCACCTGCATCAGCGTGCACCGGTGTCTGGGCGTCTTACGACCTCTGCGCTCCCTGCGCT
GGGGCCGGGCCCGCTACGCTCGCCGGGTGGCCGGGGCCGTGTGGGTGTTGGTGCTGGCCTGCC
AGGCCCCCGTGCTCTACTTTGTCACCACCAGCGCGCGCGGGGGCCGCGTAACCTGCCACGACA
CCTCGGCACCCGAGCTCTTCAGCCGCTTCGTGGCCTACAGCTCAGTCATGCTGGGCCTGCTCTT
CGCGGTGCCCTTTGCCGTCATCCTTGTCTGTTACGTGCTCATGGCTCGGCGACTGCTAAAGCCA
GCCTACGGGACCTCGGGCGGCCTGCCTAGGGCCAAGCGCAAGTCCGTGCGCACCATCGCCGT
GGTGCTGGCTGTCTTCGCCCTCTGCTFCCTGCCATTCCACGTCACCCGCACCCTCTACTACTCC
TTCCGTTCGCTGGACCTCAGCTGCCACACCCTCAACGCCATCAACATGGCCTACAAGGTTACC
CGGCCGCTGGCCAGTGCTAACAGTTGCCTTGACCCCGTGCTCTACTTCCTGGCTGGGCAGAGG
CTCGTACGCTTTGCCCGAGATGCCAAGCCACCCACTGGCCCCAGCCCTGCCACCCCGGCTCGC
TGCAGGCTGGGCCTGCGCAGATCCGACAGAACTGACATGCAGAGGATAGAAGATGTGTTGGGC
AGCAGTGAGGACTCTAGGCGGACAGAGTCCACGCCGGCTGGTAGCGAGAACACTAAGGACATT
CGGCTGTAGTTCTAGACGTCGTAG (SEQ ID NO:4)

Figure 3A

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        10         20         30         40         50
MAADLGPWND TINGTWDGDE LGYRCRFNED FKYVLLPVSY GVVCVLGLCL

        60         70         80         90        100
NAVALYIFLC RLKTWNASTT YMFHLAVSDA LYAASLPLLV YYYARGDHWP

       110        120        130        140        150
FSTVLCKLVR FLFYTNLYCS ILFLTCISVH RCLGVLRPLR SLRWGRARYA

       160        170        180        190        200
RRVAGAVWVL VLACQAPVLY FVTTSARGGR VTCHDTSAPE LFSRFVAYSS

       210        220        230        240        250
VMLGLLFAVP FAVILVCYVL MARRLLKPAY GTSGGLPRAK RKSVRTAVV

       260        270        280        290        300
LAVFALCFLP FHVTRTLYYS FRSLDLSCHT LNAINMAYKV TRPLASANSC

       310        320        330        340        350
LDPVLYFLAG QRLVRFARDA KPPTGPSPAT PARCRLGLRR SDRTDMQRIE

       360        370
DVLGSSEDSR RTESTPAGSE NTKDIRL  377  (SEQ ID NO:5)
```

Figure 3B

GAATTCCTGCAGCCCGGGGGATCCGCCCCACC**ATG**AACTGCATATCCGACTTCTTCACCTAT
GAGACCACCAAGTCGGTGGTTGTGAAGAGCTGGACCATCGGGATCATCAACCGAGTAGTTCAG
CTTCTGATCATCTCCTACTTTGTAGGGTGGGTTTTCTTGCACGAGAAGGCTACCAGGTACGGG
ACACAGCCATTGAGTCCTCGGTGGTAACCAAGGTGAAGGGCTCCGGACTCTACGCCAACAGAG
TCATGGATGTGTCTGATTACGTGACGCCACCTCAGGGCACCTCGGTCTTTGTCATCATCACCAA
GATGATGTTACTGAAAATCAGATGCAAGGATTCTGCCCAGAGAGTGAGGAGAAATACCGCTG
TGTATCAGACAGCCAGTGCGGGCCTGAGCGCTTGCCAGGTGGGGGATCCTCACTGGCCGCTG
CGTGAACTACAGCTCTGTGCTCCGGACCTGTGAGATCCAGGGCTGGTGCCCCACGGAGGTGGA
CACAGTGGAAACGCCCATCATGATGGAAGCTGAGAACTTCACTATTTCATCAAGAACAGCAT
CCGTTTCCCCCTCTTCAACTTTGAGAAGGGAAACTCCTTCCCAACCTGACAGCCAGGGACAT
GAAGACCTGCCGCTTCCACCCGGACAAGGACCCTTTCTGCCCCATCTTGCGGGTAGGGGACGT
GGTCAAGTTTGCGGGACAGGATTTTGCCAAACTGGCGCGCACGGGGGGAGTTCTGGGCATTAA
GATCGGCTGGGTGTGCGACTTGGACAAGGCCTGGGACCAGTGCATCCCCAAATACTCCTTCAC
CCGGCTCGACAGCGTTTCTGAGAAAAGCAGCGTGTCCCCAGGCTACAACTTCAGGTTTGCCAA
GTACTACAAAATGGAAAATGGCAGTGAGTACCGCACCCTCCTGAAGGCTTTTGGCATCCGCTT
CGACGTGCTGGTATACGGGAATGCTGGCAAGTTCAACATCATCCCCACCATCATCAGCTCTGT
GGCGGCCTTTACTTCTGTGGGAGTGGGAACTGTTCTCTGTGACATCATCCTGCTCAACTTCCTC
AAGGGGGCCGACCAGTACAAAGCCAAGAAGTTTGAGGAGGTGAATGAGACTACGCTGAAAATC
GCGGCTTTGACCAACCCAGTGTACCCCAGCGACCAGACCACAGCGGAGAAGCAGTCCACCGA
TTCGGGGGCCTTCTCCATAGGCCAC**TAG**GGGCTAGAGCGGCCGC (SEQ ID NO:5)

Figure 4

<u>CTACTACTACTAGGCCACGCGTCGACTAGTAC</u>GGGGGGGGGGGGGGGACCGGGGACGACCAC
CACCTACCTCCTCAGGCTGCGGCCTCGCGAGGGCCCCGGCGCGAGAGGACCCCCCTCTCCTG
AGGCCACCACTGGGCCCCCTTCTGAGTGTCCCCTGAGCACTCTCTCAGCATGAACTGCATATC
CGACTTCTTCACCTATGAGACCACCAAGTCGGTGGTTGTGAAAAGCTGGACCATCGGGATCAT
CAACCGAGTAGTTCAGCTTCTGATCATCTCCTACTTTGTAGGGTGGGTTTTCTIGCACGAGAAG
GCTTACCAGGTACGGGACACAGCCATTAAGTCCTCGGTGGTAACCAAGGTGAAGGGCTCCGGA
CTCTACACCAACAGAGTCATGGATGTGTCTGATTACGTGACGCCACCTCAGGGCACCTCGGTC
TTTGTCATCATCACCAAGATGATTGTTACTGAAAATCAGATGCAAGGATTCTGCCCAGAGAGTG
AGGAGAAATACCGCTGTGTATCAGACAGCCAGTGCGGGCCTGAGCGCTTGCCAGGGATCCTCA
CTGGCCGCTGCGTGAACTACAGCTCTGCGCTCCGGACCTGTGAGATCCAGGGCTGGTGCCCCA
CGGAGGTGGACACAGTGGAAACGCCCATCATGATGGAAGCTGAGAACTTCACTATTTTCATCA
AGAACAGCATCCGTTTCCCCCTCTTCAACTTTGAGAAGGGAAACCTCCTTCTCCAACCTGACAG
CCAGGGACATGAAGACCTGCCGCTTCCACCCGGACAAGGACCCTTCTCACCCCATCTTGCGGG
TAGGGGACGTGGTCAAGTTTGCGGGGCAGGATTTTGCCAAACTGGCGCGCACGGGGGGAGTTC
TGGGCATTAAGATCGGCTGGGTGTGCGACTTGGACAAGGCCTGGGACCAGTGCATCCCCAAAT
ACTCCTTCACCCGGCTCGACAGCGTTTCTGAGAAAAGCAGCGTGTCCCCAGGCTACAACTTCA
GGTTTGCCAAGTACTACAAAATGGAAAATGGCAGTGAGTACCGCACCCTCCTGAAGGCTTTTG
GCATCCGCTTCGACGTGCTGGTATACGGGAATGCTGGCAAGTTCAACATCATCCCCACCATCA
TCAGCTCTGTGGCGGCCTTTACTTCTGTGGGAGTGGGAACTGTTCTCTGTGACATCATCCTGCT
CAACTTCCTCAGGGGGGCCGACCAGTACAAAGCCAAGAAGTTGAGGAGGT<u>GAATGAGACTAC</u>
<u>ACTGAAAATCG</u> (SEQ ID NO:23)

Figure 5

ACCACAGTGGAGAAGCAGTCCACCGATTCGGGGGCCTTCTCCATAGGCCACTAGGGCCTCTTT

CCAGGGCCCCACACTCACAAAGGCTCCAGGCCTCCCCACAGAGGACCCTGCCTGAGCAAGGG

GGCATGGGAGGGAAGAGGGGCTCTCATTTCTGCTGCTCATTCCATGAGCATAGCTGGGACCCA

AGTGTCTGGGCCTCCGACTGCTCCAGCAGACAGGCAGTGCTCCCTGCTGAGACCCCAGTCTCA

CCTTCACTCCTTGCCTGGCCCCATCTGCTTCCTAGGACCCCTGGGGCAGGAGCACCTGAGCCA

TCCCCTTCCCAAAGAGTAGAGATTATAATGTAGGACAGATGGCCACAAGGGCCTACCAAGTGC

CAGGCACTTTCACACACGTTATCTCATTTAATCCTTAGAATAATCCTATGAGGTAGATATTAGTT

TCCCTTGTTTTGAAGATAAACCAAGGCTCAGAGAGACTGAGTCATTTGCCCCAGGCCAGATAG

CCAGGATGTGAGAGAGCTGGGATTTGAACGTCCGTCTGACTAACTCCATCGCCCACACCCCAT

GAGAGAAGATGAACTCCCAGGGTCCATCAGCCCTGCTGCTTCAGCCGCCTCCACCCTGACGGT

GATTCGGTTAATAAAGAGTAAGCCCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTAGT

CGACGCGTGGCC (SEQ ID NO:24)

FIG. 6